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(54) **Gene encoding a 30-kilodalton outer membrane protein of bordetella pertussis.**

(57) The present invention relates to a nucleotide and amino acid sequence of a 30 kilodalton outer membrane protein of Bordetella pertussis. The invention also relates to host cells and vectors comprising the nucleotide sequence, as well as a vaccine composition comprising the substantially pure protein.

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FIELD OF THE INVENTION

The present invention relates to a DNA and amino acid sequence of a 30 Kilodalton outer membrane protein of Bordetella pertussis. The protein in question is antigenic and therefore, the recombinantly produced protein may be used in vaccine compositions to protect against B. pertussis infection. It is also useful as an adjuvant in vaccine compositions against other microorganisms such as Haemophilus influenza. The isolated gene sequence also permits construction of recombinant vectors and host cells useful in producing the protein.

BACKGROUND OF THE INVENTION

The bacterium Bordetella pertussis is the causative agent of whooping cough or pertussis. It is currently routine to immunize infants and small children against B. pertussis with a vaccine comprising whole thermally or chemically inactivated B. pertussis cells. Although such vaccines are widely used and are very effective in inducing protection, such whole cell preparations necessarily contain components which are not necessary to achieve protection and which may in fact cause undesirable side effects in association with immunization. It is, therefore, preferable to identify those cellular components which are essential to immunity and utilize only those required to achieve the desired effect.

B. pertussis exhibits many proteins which are potential candidates for such a component vaccine formulation. Among these are lymphocytosis promoting factor (Morse and Morse, J. Exp. Med. 143: 1483-1502, 1976), filamentous hemagluttinin (Cowell et al, in Robbins et al, eds., Bacterial Vaccines, Thieme Stratton, Inc., N.Y., pp. 371-379); and agglutinogens (Eldering et al, J. Bacteriol. 74: 133-136, 1957). Also of recent interest are a number of virulence - associated cell envelope proteins. (Armstrong and Parker, Infect. Immun. 54: 308-314, 1986); Parker and Armstrong, Rev. Infect. Dis. 10 (Suppl. 2): S327-S330, 1988). One or more of these outer membrane components has previously been used as an adjuvant in a vaccine formulation containing Haemophilus influenzae as the active immunogen (U.S. Patent No. 4, 474,758). Outer membrane proteins also are present in an acellular pertussis vaccine produced by Takeda by copurification of several pertussis proteins.

Of particular interest to the present invention is an outer membrane protein of 30 kilodaltons. A "virulence associated doublet", referred to as Omp 30/32 has previously been described by Parton and Wardlaw. (J. Med Microbiol. 8:47-57, 1975) A 30 KD fraction of the B. pertussis outer membrane proteins was found to be particularly useful in enhancing immune response to H. influenzae capsular polysaccharide (Monji et al., Infect. Immun. 51: 865-871, 1986). Although the protein per se has been isolated, isolation depends upon chemical separation from the bacterial outer membrane and other outer membrane proteins. There has not previously been a means for producing the protein in large quantities by any other method. The present invention now makes available an alternative means for production of the 30KD protein in high yield without resort directly to the bacterial source.

SUMMARY OF THE INVENTION

The present invention provides a novel isolated gene and nucleic acid sequence encoding a 30 kilodalton outer membrane protein of Bordetella pertussis. Also provided is a complete deduced amino acid sequence of the protein.

The availability of the gene sequence of the 30KD protein permits the expression of the protein in a variety of host cells. Thus, the invention also encompasses a method for producing a purified 30KD B. pertussis outer membrane protein which comprises transforming a host cell with the 30KD gene, and culturing the host cell under conditions which permit expression of the gene in the host cell. Recovery of the protein can be made directly from the host cell, or from the culture supernatant depending upon the mode of expression in the host. Transformation of host cells may be achieved either directly by naked nucleic acid or by expression vectors engineered to carry the sequence of the 30KD protein. The invention thus also provides host cells transformed with the claimed nucleic acid sequence, as well as expression vectors comprising the sequence.

The 30KD protein is useful as the primary immunogen in a vaccine composition to provide protection against B. pertussis infection. The transformed host cells provide a convenient means for production of substantially pure (i.e., obtained free of normal cellular contaminants, and at least preferably about 90% pure) 30KD protein. Protein so produced forms the basis of a subunit vaccine comprising an effective amount of a substantially pure 30KD protein, or immunogenic portion thereof, in combination with a pharmaceutically acceptable carrier. Also encompassed by the invention is a method for immunizing an

individual against B. pertussis infection which comprises administering to an individual in need of such protection an effective amount of the aforementioned vaccine composition.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the nucleotide and predicted amino acid sequence.

Figure 2 shows expression of recombinant 30K protein in E. coli strain JM109 (DE3). Lane 1. standards, Lane 2. JM 109 (DE3) + pCLL1101 after induction, cell lysate, Lane 3. passed fraction from Affigel-blue column, Lane 4. passed fraction from DE53 column. A Coomassie Blue stained SDS-gel. B. Western blot probed with anti-sera to native 30K.

Figure 3 shows a restriction map of B. pertussis DNA fragment containing the gene for 30K outer membrane protein. The open reading frame for 30K gene is between base 770 and 1544.

Figure 4 shows a comparison of 30K and r30K protein by peptide mapping. 5 ug of native (A) or recombinant 30K (B) was loaded in each lane of 15% SDS-polyacrylamide gel with 0 ug (1), 2.5 ug (2), or 5 ug (3) endoproteinase Glu-C and digested in the gel. The gel was transferred to Nitroplus 2000 and developed with antisera to native 30K protein and protein A-Horseradish peroxidase.

DETAILED DESCRIPTION OF THE INVENTION

The DNA sequence encoding the 30KD protein is originally cloned by screening of a λ gt11 library containing genomic DNA of Bordetella pertussis. Recombinant phage that express the 30KD protein are identified by plaque lift (Mierendorf et al, Meth. Enzymol. 152: 458-469, 1987) using rabbit antisera against the 30KD protein. Positive recombinant clones are identified and phage DNA isolated. Pertussis DNA is removed and subcloned into a plasmid vector for restriction mapping, and into an M13 bacteriophage (Messing et al, Nucl. Acids Res. 9: 307, 1981) for DNA sequence analysis.

The gene containing the 30KD protein is isolated on an approximately 3.5 kb fragment of pertussis DNA. Approximately 2.5 kb is sequenced using the Sanger dideoxy termination method (PNAS USA 74: 5463-5467, 1977) from both single stranded M13 and double stranded plasmid subclones, generated by exonuclease III deletion subcloning methods. The DNA sequence of the 30KD protein is presented in Figure 1.

The recombinant protein consists of a sequence of 242 amino acids, also shown in Figure 1. The protein is expressed in E. coli using the T7 RNA polymerase and promoter system (Studier et al., Meth. Enzymol. 185: 60-69, 1990). The open reading frame encoding the 30KD protein is cloned into a pGEM 7Zf+ plasmid behind a T7 RNA polymerase promoter. The resulting plasmid is designated pCLL 1101. The plasmid is transformed into E. coli strain JM109 (DE3) containing the T7 RNA polymerase gene under the control of the lac UV5 promoter. Expression of the T7 RNA polymerase is induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG). The presence of the 30KD protein is confirmed by Western blotting of whole cell lysates, shown in Figure 2B.

The expressed protein is purified from lysates of IPTG - induced bacterial cultures. The protein obtained after a two - step column chromatography purification is approximately 90% pure. The recombinant purified protein from E. coli is compared to the 30KD native purified protein from B. pertussis. The native and recombinant proteins have the same apparent molecular weight as determined by SDS-PAGE, the same isoelectric point (about 9) as determined by isoelectric focusing, both cross-react with anti-30KD antisera, and both have the same peptide mapping pattern when digested with endoproteinase Glu-C.

The following examples illustrate the cloning and expression of the 30KD protein gene in a T7 RNA polymerase expression system. However, although this T7 expression system has proven quite efficient, it is to be understood that this is not the only means by which 30 KD protein can be produced recombinantly. Production of the protein can be achieved by incorporation of the gene into any suitable expression vector and subsequent transformation of an appropriate host cell with the vector; alternately the transformation of the host cells can be achieved directly by naked DNA without the use of a vector. Production of the protein by either eukaryotic cells or prokaryotic cells is contemplated by the present invention. Examples of suitable eukaryotic cells include mammalian cells, plant cells, yeast cells and insect cells. Similarly, suitable prokaryotic hosts, in addition to E. coli, include Bacillus subtilis.

Other suitable expression vectors may also be employed and are selected based upon the choice of host cell. For example, numerous vectors suitable for use in transforming bacterial calls are well known. For example, plasmids and bacteriophages, such as λ phage, are the most commonly used vectors for bacterial hosts, and for E. coli in particular. In both mammalian and insect cells, virus vectors are frequently used to obtain expression of exogenous DNA. In particular, mammalian cells are commonly transformed with SV40

or polyoma virus; and insect cells in culture may be transformed with baculovirus expression vectors. Yeast vector systems include yeast centromere plasmids, yeast episomal plasmids and yeast integrating plasmids.

It will also be understood that the practice of the invention is not limited to the use of the exact sequence of the gene as defined in Figure 1. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce silent changes in the resulting protein molecule are also contemplated. For example, alteration in the gene sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, are also expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. Therefore, where the phrase "30KD protein DNA sequence" or "30KD protein gene" is used in either the specification or the claims, it will be understood to encompass all such modifications and variations which result in the production of a biologically equivalent 30 KD protein. In particular, the invention contemplates those nucleic acid sequences which are sufficiently duplicative of the sequence of Figure 1 so as to permit hybridization therewith under standard high stringency southern hybridization conditions, such as those described in Maniatis et al., (Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, 1989), or encode proteins which react with antisera to native 30KD protein.

In addition to a full length gene and protein, the invention encompasses fragments of each. In particular, the invention encompasses nucleic acid fragments encoding peptides, and the peptides per se, which retain the antigenicity of the parent molecule. Preferably the fragments in question encode peptides containing epitopes which elicit production of protective antibodies. In addition to preparation by recombinant methods, such smaller peptides can also be prepared synthetically by known peptide synthesis techniques.

The gene product in purified form, or a synthetic immunogenic peptide is useful in the preparation of a vaccine composition for prevention of pertussis. Either the whole protein, or any active portion thereof, can be employed as an immunogenic agent in such a composition. The protein prepared by recombinant methods can be isolated from host cells by standard protein isolation techniques. The purified protein is then combined with any of the commonly used acceptable carriers such as water, physiological saline, ethanol, polyols, such as glycerol or propylene glycol, or vegetable oils, as well as any of the vaccine adjuvants known as the art. The proteins may also be incorporated into liposomes for use in a vaccine preparation. As used herein "pharmaceutically acceptable carriers" is to encompass any and all solvents, dispersion media, coatings and antifungal agents, isotonic and absorption delaying agents and the like. Except insofar as any conventional medium is incompatible with the active ingredient, its use in the therapeutic composition is contemplated.

In addition to its use as the sole active agent in a vaccine composition, the 30KD protein, or active portions thereof, may also be combined with other active agents. For example, a pertussis vaccine may comprise the 30KD protein with one or more purified and isolated outer membrane proteins, or other known immunogenic proteins from Bordetella pertussis. Moreover, the 30KD protein may be combined as an active component with immunogenic agents against other infectious diseases, such as influenza, hepatitis, or herpes. Also, the 30KD protein may be used in vaccine compositions, in adjuvant effective amounts, to improve the immune response to other immunogenic agents, such as those noted above.

The microorganisms and other biological materials referred to herein are retained in the collections of American Cyanamid Company, Lederle Laboratories, Pearl River, New York, and E. coli strain JM109 (DE 3) containing plasmid pCLL1101, has been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, on September 18, 1990, as ATCC 68402.

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

1. Cloning of 30KD gene from λgt11 library

Genomic DNA is isolated from B. pertussis strain 130. EcoRI linkers are added to mechanically sheared DNA and then cloned into the EcoRI site of λgt11. The library contains approximately 1.6 X 10^6 independent clones. The library is diluted 1:10^5, for each 150 mm plate, 0.1 ml is mixed with 0.6 ml of E.

coli strain Y1090 and incubated at room temperature for 20 min. The cells are plated in 7.5 ml LB top agar on LB plates and incubated for 3 hr at 42°C. Nitrocellulose filters are soaked in 10mM IPTG and air dried. These are laid on the plates which are incubated at 37°C for 3 hr. The filters are blocked with 10mM Tris-HC1, pH 8.0, 150 mM NaCl, 0.05% Tween 20 (TBST) plus 1% bovine serum albumin (BSA) overnight. The filters are washed in TBST and anti-30K sera is added. Following a 60 min incubation the filters are washed again with TBST, then incubated with Protein A - Horseradish Peroxidase conjugate for 60 min. The filters are washed in TBS (10mM Tris-HC1, ph 8.0, 150mM NaCl) and then incubated in the presence of the Horseradish peroxidase substrate (4-chloro-1-napthol and hydrogen peroxide in TBS). Positive plaques are picked and eluted into SM (.IM NaCl, 10mM MgS04, 50mM Tris HCl, pH7.5, 0.01% gelatin) buffer. Positive phage are plaque purified by repeating the screening procedure.

2. Sequencing of the 30KD gene

Approximately 3.6kb fragment of pertussis DNA is isolated from positive λ clones. Two EcoRI fragments (1.4 and 2.2 kb) are subcloned into M13mp18 for sequencing by the dideoxytermination method. Exonuclease III deletion subclones are generated to sequence overlapping subclones (Henikoff, S. (1984) Gene 28: 357). The EcoRI site is located in the middle of the open reading frame. To confirm the sequence across this junction, plasmid clones containing the entire open reading frame are sequenced using Sequenase (US Biochemicals). Since pertussis DNA has a high G + C content, regions of compression are sequenced in both directions. A restriction map of the 30KD protein gene is provided in Figure 3. Comparison of this map with that shown in Shareck and Cameron (J. Bacteriol. 159: 780-782, 1984; Fig. 2) shows that the gene of the present invention does not encode the 30Kd protein disclosed by these authors.

3. Expression and purification of recombinant 30KD Protein

Pertussis DNA is isolated from positive phage and subcloned into pGEM7zf+ for expression. A 3kb KpnI - SacI fragment of pertussis DNA is cloned into pGEM7zf+ after the T7 RNA polymerase promoter (designated pCLL 1101) and transformed into the E. coli host strain JM109 (DE3) which contains the T7 RNA polymerase gene under the control of the lacUV5 promoter. Cultures of JM109 (DE3) containing pCLL 1101 are grown in LB plus ampicillin (50 µg/ml) at 37°C to an OD of 1 at 550nm. IPTG is added to a final concentration of 0.5mM and cultures incubated for an additional 3 hr. Cells are harvested by centrifugation 5,000xg for 10 minutes and washed with water. The cell pellet is resuspended in 10ml lysis buffer (50mM Tris-HC1, pH8.0, 1mM EDTA, 100mM NaCl), 0.3ml lysozyme (10 mg/ml in lysis buffer) is added and the mixture incubated at room temperature for 30 min. As the viscosity increases 0.07ml DNase (1 mg/ml in lysis buffer) is added. The mixture is centrifuged at 15,000xg for 30 min at 4°C. The supernatant is centrifuged at 200,000xg for 30 min. The supernatant fraction is passed over an Affigel Blue column in 50mM Tris-HC1, pH 7.4. The flow through fraction is collected and passed over a DE53 column in 50mM Tris-HC1, pH 8.0. These two chromatography steps result in a preparation of recombinant 30KD protein which is approximately 90% pure.

4. Characterization of the recombinant 30KD protein

Purified 30KD protein from B. pertussis is compared to the recombinant protein purified from E. coli by several methods. The proteins when fractionated by SDS-PAGE on a 12.5% acrylamide gel migrate to identical apparent molecular weight. Western blot analysis shows the proteins both cross-react with antisera against the native 30KD protein. In addition, the native and recombinant protein focus at a pI of approximately 9 in isoelectric focusing gels. The predicted pI of the mature protein based on the amino acid sequence deduced from the DNA sequence is 9.8.

In order to confirm the identity of the recombinant protein, peptide mapping is done as described by D.W. Cleveland (Meth. Enzymol., vol 96, p. 222-229, 1983). Approximately 10 µg protein is loaded on a 15% polyacrylamide gel in the presence of increasing amounts of endoproteinase Glu-C (0, 2.5, 5 µg) in digestion buffer consisting of 50mM Tris-HC1, pH 6.8, 10% glycerol and 0.1% SDS. The samples are electrophoresed into the stacking gel and current turned off for 1 hr to allow digestion. The current is turned on and resulting peptides separated. One gel is stained with Coomassie Brilliant Blue and a second electrotransferred to nitrocellulose membrane for Western blot analysis. Both the native and recombinant protein have the same peptide digestion pattern. One difference that is observed between the native and recombinant proteins is the native protein has a blocked amino-terminus. This is not the case with the recombinant 30K protein, where the first 50 amino acids have been determined by protein sequencing.

Sequence ID No.: 1

Sequence Type: Nucleic Acid and Amino Acid

Sequence Length: 960 Base Pairs, 257 Amino Acids

Strandedness: Single

Topology: Linear

Original Source Organism: <u>Bordetella</u> <u>pertussis</u>

Features: DNA (genomic) and Protein

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CAGGATTTGC TCCCATATCC CATTCATGCA CTTGCGCTGG          40

ATGCGCAAGC ACCCTCTCCA GACAACGCCA AGTAAACATT          80

CAAAAGGTCA AAGGACATAC                               100

ATG AAA CGC ATC GCC ATG CTG GCT GCT GCC TGC GTC     136
Met Lys Arg Ile Ala Met Leu Ala Ala Ala Cys Val
 1              5                   10

ATT GCC GTG CCC GCT TTC GCC CAG AAC GTG GCG ACC     172
Ile Ala Val Pro Ala Phe Ala Gln Asn Val Ala Thr
        15                  20

GTG AAC GGC AAG CCC ATT ACT CAG AAG AGC CTG GAT     208
Val Asn Gly Lys Pro Ile Thr Gln Lys Ser Leu Asp
 25                 30                  35

GAG TTC GTC AAG CTG GTC GTC AGC CAG GGC GCT ACC     244
Glu Phe Val Lys Leu Val Val Ser Gln Gly Ala Thr
            40                  45

GAT TCG CCC CAG CTG CGT GAG CAG ATC AAG CAG GAA     280
Asp Ser Pro Gln Leu Arg Glu Gln Ile Lys Gln Glu
    50                  55                  60

ATG ATC AAC CGC CAG GTG TTC GTG CAG GCG GCC GAG     316
Met Ile Asn Arg Gln Val Phe Val Gln Ala Ala Glu
                65                  70

AAG GAC GGC GTC GCC AAG CAG GCC GAC GTG CAG ACT     352
Lys Asp Gly Val Ala Lys Gln Ala Asp Val Gln Thr
        75                  80

GAG ATC GAG CTG GCC CGC CAC GGA GTC CTG GTG CGC     388
Glu Ile Glu Leu Ala Arg His Gly Val Leu Val Arg
 85                 90                  95
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GCC CTG ATG GCC GAC TAC CTG CAA AAA CAC CCC GTC      424
Ala Leu Met Ala Asp Tyr Leu Gln Lys His Pro Val
            100                 105

ACC GAC GCC CAG GTC AAG GCC GAA TAC GAA AAG ATC      460
Thr Asp Ala Gln Val Lys Ala Glu Tyr Glu Lys Ile
    110                 115                 120

AAG AAA GAA CAG GCC GGC AAG ATG GAA TAC AAG GTC      496
Lys Lys Glu Gln Ala Gly Lys Met Glu Tyr Lys Val
                125                 130

CGT CAC ATC CTG GTC GAG GAC GAA AAG ACG GCC AAC      532
Arg His Ile Leu Val Glu Asp Glu Lys Thr Ala Asn
        135                 140

GAC CTG CTG GCC CAG GTC AAG AGC AAC AAG AAC AAG      568
Asp Leu Leu Ala Gln Val Lys Ser Asn Lys Asn Lys
145                 150                 155

TTC GAC GAT CTG GCC AAG AAG AAC TCC AAG GAC CCC      604
Phe Asp Asp Leu Ala Lys Lys Asn Ser Lys Asp Pro
            160                 165

GGC AGC CCG AGC GCG GCG GCG ACC TGG GTT GGG CGC      640
Gly Ser Pro Ser Ala Ala Ala Thr Trp Val Gly Arg
    170                 175                 180

TGC ACC AAC TAC GTC CAG CCG TTT GCC GAG GCC GTG      676
Cys Thr Asn Tyr Val Gln Pro Phe Ala Glu Ala Val
                185                 190

ACC AAG CTG AAG AAG GGC CAA CTG GTC GAC AAG CCG      712
Thr Lys Leu Lys Lys Gly Gln Leu Val Asp Lys Pro
        195                 200

GTG CAG ACC CAG TTC GGC TGG CAC GTG ATC CAG GTC      748
Val Gln Thr Gln Phe Gly Trp His Val Ile Gln Val
205                 210                 215

GAC GAT ACC CGT CCG GTC GAA TTC CCC GCC ATG GAC      784
Asp Asp Thr Arg Pro val Glu Phe Pro Ala Met Asp
            220                 225

CAG GTG CGC CCG CAA CTG GAA GAA ATG CTG CGC CAG      820
Gln val Arg Pro Gln Leu Glu Glu Met Leu Arg Gln
    230                 235                 240

CAA ACC CTG GCC AAC TAC CAG AAG CAA TTG CGC GAA      856
Gln Thr Leu Ala Asn Tyr Gln Lys Gln Leu Arg Glu
                245                 250
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CAG GCC AAG ATC CAG                                     871
Gln Ala Lys Ile Gln
        255     257

TAAGCGCCAA GCCATCGCCA TCAACAAAAT TGCCCGCTTT             911

CGCGGGAATT TTGTTTTCGG CTGCCGGGCG CCGGCGCCGC             951

TTCGCCTAA                                               960
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Claims

1. An isolated nucleic acid sequence encoding a 30KD outer membrane protein of Bordetella pertussis.

2. The sequence of Claim 1 in which the full-length protein has an isoelectric point of about 9.

3. The sequence of Claim 1 which comprises the sequence depicted in Figure 1, or a fragment thereof encoding a biologically active peptide.

4. The sequence of Claim 1 which is hybridizable with the sequence depicted in Figure 1 under standard high stringency conditions.

5. An isolated 30kd outer membrane protein of Bordetella pertussis comprising the sequence depicted in Figure 1, or a biologically active fragment thereof.

6. The protein or fragment of Claim 5 which is immunogenic.

7. A method of producing a substantially pure 30 kd outer membrane protein of Bordetella pertussis which comprises transforming a host cell with the gene of Claim 1, and culturing the host cell under conditions which permit expression of the gene by the host cell.

8. An expression vector comprising the gene of Claim 1.

9. A host cell transformed with the gene of Claim 1.

10. A vaccine composition comprising an immunogenically effective amount of a substantially pure 30KD outer membrane protein of Bordetella pertussis, or active fragments thereof, in combination with a pharmaceutically acceptable carrier.

11. The composition of Claim 16 which comprises other Bordetella pertussis antigens.

12. A vaccine composition comprising an adjuvant effective amount of a substantially pure 30KD outer membrane protein of Bordetella pertussis, in combination with at least one non-Bordetella pertussis antigen, and a pharmaceutically acceptable carrier.

13. A method of protecting an individual against Bordetella pertussis infection comprising administering to the individual an immunogenically effective amount of a substantially pure 30KD membrane protein of Bordetella pertussis.

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CAGGATTTGCTCCCATATCCCATTCATGCACTTGCGCTGGATGCGCAAGCACCCTCTCCA  60

GACAACGCCAAGTAAACATTCAAAAGGTCAAAGGACATACATGAAACGCATCGCCATGCT 120
                                              MetLysArgIleAlaMetLeu
                                                         -15

GGCTGCTGCCTGCGTCATTGCCGTGCCCGCTTTCGCCCAGAACGTGGCGACCGTGAACGG 180
 AlaAlaAlaCysValIleAlaValProAlaPheAlaGlnAsnValAlaThrValAsnGly
      -10            -5                1             5

CAAGCCCATTACTCAGAAGAGCCTGGATGAGTTCGTCAAGCTGGTCGTCAGCCAGGGCGC 240
 LysProIleThrGlnLysSerLeuAspGluPheValLysLeuValValSerGlnGlyAla
      10             15             20             25

TACCGATTCGCCCCAGCTGCGTGAGCAGATCAAGCAGGAAATGATCAACCGCCAGGTGTT 300
 ThrAspSerProGlnLeuArgGluGlnIleLysGlnGluMetIleAsnArgGlnValPhe
      30             35             40             45

CGTGCAGGCGGCCGAGAAGGACGGCGTCGCCAAGCAGGCCGACGTGCAGACTGAGATCGA 360
 ValGlnAlaAlaGluLysAspGlyValAlaLysGlnAlaAspValGlnThrGluIleGlu
      50             55             60             65

GCTGGCCCGCCACGGAGTCCTGGTGCGCGCCCTGATGGCCGACTACCTGCAAAAACACCC 420
 LeuAlaArgHisGlyValLeuValArgAlaLeuMetAlaAspTyrLeuGlnLysHisPro
      70             75             80             85

CGTCACCGACGCCCAGGTCAAGGCCGAATACGAAAAGATCAAGAAAGAACAGGCCGGCAA 480
 ValThrAspAlaGlnValLysAlaGluTyrGluLysIleLysLysGluGlnAlaGlyLys
      90             95             100            105

GATGGAATACAAGGTCCGTCACATCCTGGTCGAGGACGAAAAGACGGCCAACGACCTGCT 540
 MetGluTyrLysValArgHisIleLeuValGluAspGluLysThrAlaAsnAspLeuLeu
      110            115            120            125

GGCCCAGGTCAAGAGCAACAAGAACAAGTTCGACGATCTGGCCAAGAAGAACTCCAAGGA 600
 AlaGlnValLysSerAsnLysAsnLysPheAspAspLeuAlaLysLysAsnSerLysAsp
      130            135            140            145

CCCCGGCAGCCCGAGCGCGGCGGCGACCTGGGTTGGGCGCTGCACCAACTACGTCCAGCC 660
 ProGlySerProSerAlaAlaAlaThrTrpValGlyArgCysThrAsnTyrValGlnPro
      150            155            160            165
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FIGURE 1A

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GTTTGCCGAGGCCGTGACCAAGCTGAAGAAGGGCCAACTGGTCGACAAGCCGGTGCAGAC 720
 PheAlaGluAlaValThrLysLeuLysLysGlyGlnLeuValAspLysProValGlnThr
      170                 175                 180                 185

CCAGTTCGGCTGGCACGTGATCCAGGTCGACGATACCCGTCCGGTCGAATTCCCCGCCAT 780
 GlnPheGlyTrpHisValIleGlnValAspAspThrArgProValGluPheProAlaMet
      190                 195                 200                 205

GGACCAGGTGCGCCCGCAACTGGAAGAAATGCTGCGCCAGCAAACCCTGGCCAACTACCA 840
 AspGlnValArgProGlnLeuGluGluMetLeuArgGlnGlnThrLeuAlaAsnTyrGln
      210                 220                 225                 230

GAAGCAATTGCGCGAACAGGCCAAGATCCAGTAAGCGCCAAGCCATCGCCATCAACAAAA 900
 LysGlnLeuArgGluGlnAlaLysIleGln
      235                 240

TTGCCCCGCTTTCGCGGGAATTTTGTTTTCGGCTGCCGGGCGCCGGCGCCGCTTCGCCTAA 960
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FIGURE 1B

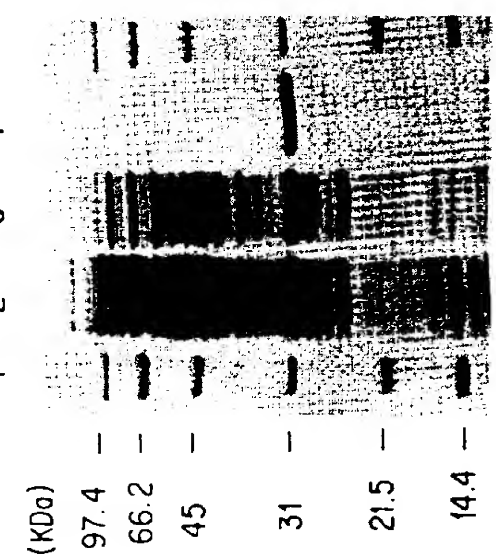


FIG.2A

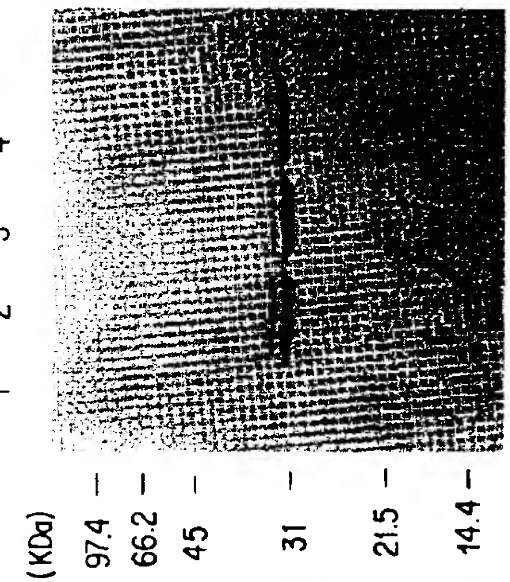
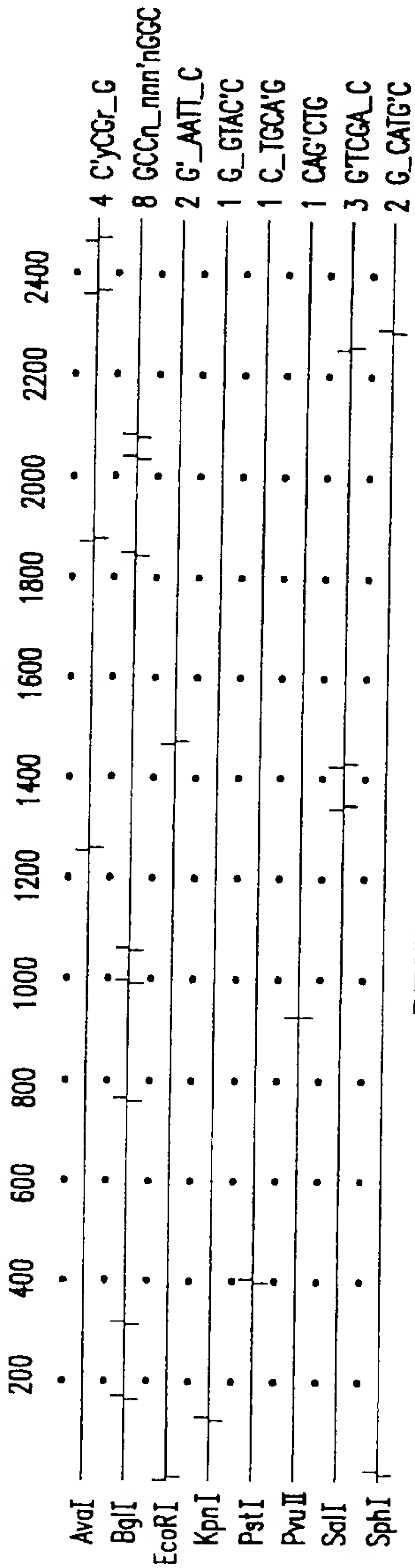


FIG.2B



ENZYMES THAT DO NOT CUT:

BglII

FIG.3

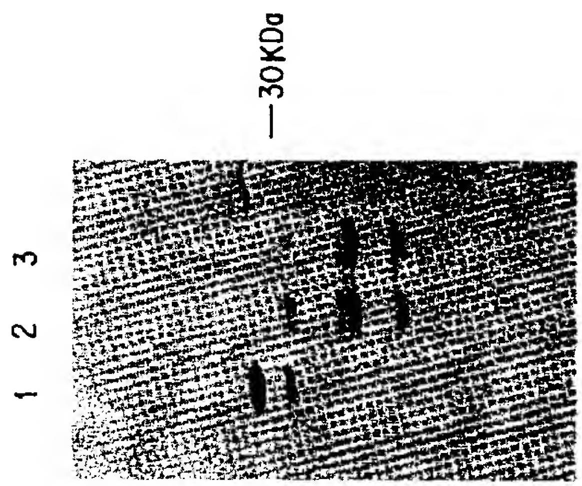


FIG.4B

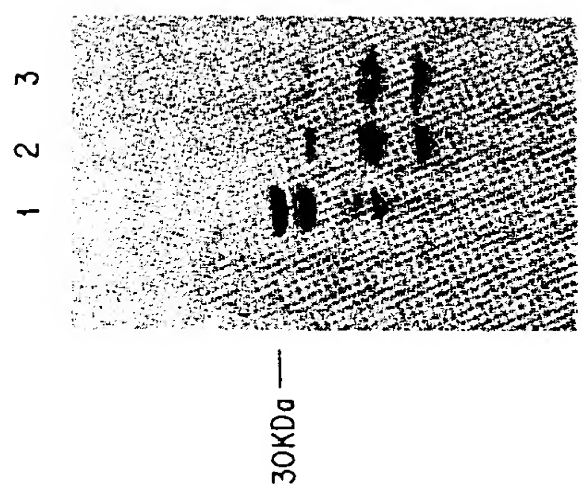


FIG.4A